Carotenoid scarcity, synthetic pteridine pigments and the evolution of sexual coloration in guppies (*Poecilia reticulata*)

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Carotenoid-based sexual coloration is the classic example of an honest signal of mate quality. Animals cannot synthesize carotenoid pigments and ultimately depend on dietary sources. Thus, in carotenoid-poor environments, carotenoid coloration may be a direct indicator of foraging ability and an indirect indicator of health and vigour. Carotenoid coloration may also be affected, more directly, by parasites in some species. Carotenoids are not, however, the only conspicuous pigments available to animals. Pteridine pigments, with similar spectral properties, are displayed in the exoskeletons and wings of insects, the irides of birds and the skins of fishes, lizards and amphibians. Unlike carotenoids, pteridines are synthesized *de novo* by animals. We report that the orange spots that male guppies (*Poecilia reticulata*) display to females contain red pteridine pigments (drosopterins) in addition to carotenoids. We also examined the relationship between drosopterin production by males and carotenoid availability in the field. The results contrasted sharply with the hypothesis that males use drosopterins to compensate for carotenoid scarcity: males used more, not less, drosopterins in streams with higher carotenoid availability. The positive association between drosopterin use and carotenoid availability could reflect the costs of drosopterin synthesis or it could be a consequence of females preferring a particular pigment ratio or hue. Male guppies appear to use drosopterin pigments in a manner that dilutes, but does not eliminate, the indicator value of carotenoid coloration.

Keywords: coloration; sexual selection; carotenoid availability; pteridine pigments; condition-dependent indicators; *Poecilia reticulata*

1. INTRODUCTION

Most of the conspicuous yellow, orange and red colours used in intra- and interspecific communication are carotenoid based, and carotenoids are also involved in crypsis (Needham 1974; Rothchild *et al*. 1975, 1986; Fox 1976; Goodwin 1984). Some carotenoids may provide animals with physiological benefits, not only as precursors for vitamin A but also as antioxidants, free-radical scavengers, immune-system enhancers or stimulants and cancer inhibitors (Bendich 1989, 1993; Burton 1989; Connett *et al*. 1989; Krinsky 1989, 1998; Mayne 1996). Surprisingly, animals have lost the ability to synthesize these pigments *de novo* (Goodwin 1984; Schiedt 1989). Some insects derive carotenoids from microbial symbionts (Kayser 1985) but most animals obtain carotenoids exclusively from food. Hence, carotenoids may be a limiting resource for animals in some environments (Brush & Power 1976; Endler 1980; Slagsvold & Lifjeld 1985; Hill 1993; Gray 1996; Grether *et al*. 1999).

Endler (1980) suggested that the carotenoid-containing orange spots of male guppies (*Poecilia reticulata*) may be indicators of foraging ability, which females could use to choose high-quality mates. Subsequent studies confirmed that the level of carotenoids in the diet affects the chroma (colour saturation) of the orange spots (Kodric-Brown 1989; Grether 2000), that female guppies prefer males with higher orange chromas (Kodric-Brown 1989, 1993;

Houde & Torio 1992; Grether 2000) and that some field populations of guppies are carotenoid limited (Grether *et al*. 1999). The chroma of the orange spots has also been shown to be reduced by parasitic infection (Houde & Torio 1992) and to correlate positively with swimming performance, a measure of condition (Nicoletto 1991). Variations of Endler's hypothesis have been applied to several other species with carotenoid coloration (reviewed in Olson & Owens 1998), most notably the house finch (e.g. Hill 1990, 1991; Hill & Montgomerie 1994; but see Hudon 1994; Thompson *et al*. 1997 and Zahn & Rothstein 1999). Carotenoid coloration may also more directly reveal a male's history of disease, current state of health or potential to respond to future infections through the physiological effects of parasites on carotenoid metabolism or the use and oxidation of carotenoids by the immune system (Kodric-Brown & Brown 1984; Putnam 1992; Hudon 1994; Lozano 1994; Thompson *et al*. 1997; Olson & Owens 1998; Wedekind *et al*. 1998; Camplani *et al*. 1999; Von Schantz *et al*. 1999; Møller *et al*. 2000).

Although females may benefit from the condition dependence of male carotenoid coloration, it would be advantageous for males to be optimally conspicuous regardless of their condition or the availability of carotenoids in the environment. From the perspective of an evolutionarily stable strategy, carotenoid-limited populations would seem to be vulnerable to invasion by mutants displaying synthesized pigments mimicking the spectral properties of carotenoids. Synthesizable alternatives to carotenoids do exist. Yellow, orange and red pteridine

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pigments are found in numerous vertebrate and invertebrate taxa, including crustaceans, insects, fishes, amphibians, lizards and birds (Bagnara & Hadley 1973; Needham 1974; Fox 1976; Henze *et al.* 1977; Oliphant & Hudon 1993). In some species, carotenoids and pteridines are found together in individual pigment cells (e.g. *Xiphophorus helleri*; Goodrich *et al.* 1941) or in the same cell types at different stages of development (pteridines in juveniles, carotenoids in adults; Hama 1970; Bagnara & Hadley 1973). Pteridines are derived from purines, which are synthesized *de novo* by animals from carbohydrates and proteins (Hurst 1980). This presents an evolutionary paradox: how could the honesty of carotenoid-based sexual coloration be maintained over evolutionary time in the presence of synthesizable alternatives? What prevents males from enhancing their attractiveness and 'deceiving' females (or rival males; Evans & Norris 1996) with pteridines?

We have found that the orange spots of male guppies contain red pteridines (drosopterins) in addition to carotenoids and that the amount of drosopterins in the spots varies geographically. Both carotenoids and drosopterins contribute positively to the chroma of the orange spots (G. F. Grether, M. Cummings and J. Hudon, unpublished data); in contrast, the total area of orange on a male is highly heritable (Winge 1927; Haskins *et al.* 1961; Houde 1992) and is not affected by the level of carotenoids in the diet (Kodric-Brown 1989; Grether 2000). Our initial hypothesis was that drosopterins function as carotenoid substitutes or mimics, which males produce to compensate for a lack of carotenoids in the diet. An underlying assumption is that orange-spot chroma has both costs and benefits, and therefore an optimal level. In this paper, we test the carotenoid-mimicry hypothesis by examining the relationship between the drosopterin content and the carotenoid content of the orange spots across an environmental gradient in carotenoid availability. The simplest prediction is that the mean drosopterin content of the orange spots should be negatively correlated, across populations, with the mean carotenoid content.

2. METHODS

(a) *Study species and populations*

Guppies are small teleost fishes native to the tropical-forest streams of Trinidad (West Indies) and nearby regions (Endler 1978). The primary carotenoid source for guppies is attached unicellular algae, the availability of which (in undisturbed streams) is largely a function of stream order. Low-order streams tend to be narrower, make smaller gaps in the forest, receive less photosynthetically active light and contain smaller standing crops of algae, but not lower densities of guppies, than high-order streams (Grether *et al.* 2001). Consequently, guppy populations in the upper reaches of a drainage basin tend to experience greater carotenoid limitation than those in the lower reaches (Grether *et al.* 1999).

Guppies were sampled from two streams in each of three river systems (Marianne, Paria and Quare) in the Northern Range of Trinidad (Universal Transverse Mercator Grid coordinates: PS 858 895, PS 842 894, PS 911 920, PS 895 907, PS 970 806 and PS 969 809). We selected streams that were separated from each other by barriers to guppy dispersal, in intact old-growth forest and above waterfalls that excluded

predatory fishes except for the minor predator *Rivulus hartii*. These restrictions eliminated predation and anthropogenic disturbance as potentially confounding variables and ensured that the study streams contained genetically independent populations of guppies (for further details on these streams see Grether *et al.* (2001)). Previous work on these six streams showed that guppies ingest carotenoids at lower rates, that males have less carotenoids in their orange spots and that there is a trade-off between orange-spot area and carotenoid concentration in the streams with the lower carotenoid availabilities (Grether *et al.* 1999).

(b) *Skin-pigment analyses*

Males collected for skin-pigment analyses were photographed under anaesthetic (ethyl 3-aminobenzoate methane sulphonic acid salt (MS-222), Sigma-Aldrich Co., St Louis, MO, USA) within hours of field capture, frozen in liquid nitrogen and stored at $-80\,^\circ$C. Orange body spots were measured from slides using image-analysis software (Houde & Endler 1990) and summed to give the orange area. Out of a random sample of between 36 and 41 males per stream, we selected 15 per stream for skin-pigment analysis, with the goal of pairing males with the most similar orange areas between streams while sampling the full range of orange areas within streams. The skin, excluding the head and fins, was stripped from the body with surgical instruments, divided into an orange-spot fraction and a non-orange-spot fraction and allowed to dry for a few minutes. The carotenoids in each skin fraction were extracted with acetone, transferred to a new vial, concentrated under a flow of nitrogen to remove the acetone and redissolved in hexane. Skin pteridines were extracted from the carotenoid-stripped skin with 30% acidified (with HCl to pH 2) ethanol overnight (Ephrussi & Herold 1944).

Carotenoid concentration was determined from absorbance of extracts at the peak of absorption (437–446 nm) in hexane using an extinction coefficient, $E_{1\,cm}^{1\%}$, of 2350 (Britton 1985) on a Beckman DU-65 spectrophotometer (Beckman-Coulter Inc., Fullerton, CA, USA). Absorption spectra were also recorded from 350 to 550 nm. Coloured pteridines were quantified at the peak of absorption (478–495 nm) and absorption spectra were recorded from 400 to 600 nm.

Skin pteridines were characterized chromatographically as well as spectrally and the colour of the pigment fluorescence on chromatograms was recorded. Thin-layer chromatography was performed in two dimensions to maximize band separation, using precoated plates of cellulose with fluorescent indicator (Chromagram sheets, Eastman Kodak Co., Rochester, NY, USA). The ethanol extracts were concentrated slightly under a stream of nitrogen applied 1 inch from the bottom and left sides of the plate, and dried with hot air from a hair dryer. Pigment bands were separated with 1 M acetic acid (first dimension, 4 h) and 1-propanol: 2% ammonium acetate in water (2:1) (second dimension, 16 h) (Schwinck 1975). Extracts from the orange spots of individual fishes from the Marianne and Paria river systems were sufficiently intense in colour to be run individually but the Quare extracts had to be pooled (within streams) to obtain sufficient material for pigment identification by thin-layer chromatography. Colourless pteridines were detected with a Burton 9312 long-wave hand lamp. A variety of pteridine standards were used, including 6-biopterin, isoxanthopterin, leucopterin, pterin-6-carboxylic acid, pterine, xanthopterin (Sigma Chemical Co., Sigma-Aldrich Co., St Louis, MO, USA) and sepiapterin (Dr B. Schircks Laboratories, Jona, Switzerland).

Drosopterins (drosopterin, isodrosopterin and neodrosopterin) were extracted from the heads of wild fruit flies (*Drosophila* spp.) using the procedures described above.

(c) Gut-pigment analyses

Fishes collected for gut-content analysis were frozen instantly after capture in the field, to stop gut-pigment absorption, and stored at $-80\,^{\circ}$C. Carotenoids were extracted from the first third of the gut (roughly equivalent to the stomach) of between six and eight fishes of each sex per stream, using acetone. The acetone extracts were filtered (Cameo 13N syringe filters, nylon, 0.45 micron; Micron Separations Inc., Westboro, MA, USA), evaporated under a flow of nitrogen and redissolved in the high-performance-liquid-chromatography mobile phase. High-performance liquid chromatography was carried out with a Waters instrument (Waters Inc., Milford, MA, USA) equipped with two Waters 501 pumps, a 712 WISP^{TM} auto-injector, a System Interface Module, a Lambda Max 481 UV detector and a 420AC fluorescence detector. Carotenoid pigments were eluted isocratically on a Zorbax octadecyl-siloxane (Du Pont Company, Wilmington, DE, USA) reversed-phase column (internal diameter of 4.6 mm, length 25.0 cm) with a mixture of acetonitrile–methanol–dichloromethane (41:50:9) flowing at $1\,\mathrm{mL\,min^{-1}}$ (De Leenheer & Nelis 1992). Peak detection was at 450 nm. β-carotene eluted later than expected and was not measured in Paria fishes; only lutein–zeaxanthin data were used in the analysis. The results for Marianne and Quare were qualitatively the same with β-carotene included.

(d) Statistical details

We tested for stream differences in the drosopterin content of the orange spots using paired *t*-tests in which males with similar orange areas were paired ($n=8$, 12 and 14 pairs in the Marianne, Paria and Quare river systems, respectively) to eliminate the potentially confounding effects of stream differences in mean orange area. Other statistical analyses used the full sample of 15 males per stream. Drosopterin mass was $\log_{10}$-transformed to minimize the departure from parametric assumptions of normality and homoscedasticity. However, the results were qualitatively the same (at $\alpha = 0.05$) with the untransformed data and also when Wilcoxon and Kruskal–Wallis tests were used instead of *t*-tests and ANOVA (not shown). Foregut carotenoid mass was $\log_{10}$ transformed and the stream means were adjusted using ANCOVA to remove the effects of body mass and sex. We tested for variation between streams in the positions of the absorption maxima of drosopterins using the Kruskal–Wallis test because these data could not be transformed to meet parametric assumptions.

3. RESULTS

(a) Pigment characterization

Two types of pigment were present in the integument of the guppies examined. Yellow hydrophobic compounds (carotenoids) were present in both the orange-spot and non-orange-spot fractions, while red hydrophilic compounds (drosopterins) were present solely in the orange-spot fraction of males. Coloured pteridines were completely absent from the integuments of females. The carotenoids in the integuments of guppies from all streams were mostly esters of tunaxanthin, with average peak absorption ranging from 438 to 442 nm and being highest in the orange spots (J. Hudon and G. F. Grether, unpublished data).

The ethanol extracts of the orange-spot fraction of male guppies all displayed identical absorption spectra, characterized by a broad peak in the visible region centred at about 478–495 nm (mean $\pm$ s.e.m. $= 479.88 \pm 0.25$, $n = 90$). The position of the peak did not vary significantly between streams ($p = 0.4$). Two-dimensional chromatography resolved several reddish spots in guppies from all streams. The pattern that was produced precisely matched the fingerprint pattern produced by the three most abundant drosopterins in *Drosophila* eye extracts, specifically drosopterin, isodrosopterin and neodrosopterin (Schwinck 1975). Colourless pteridines were present as well, notably isoxanthopterin and biopterin (and an unidentified pigment, probably ranachrome-3, according to Henze *et al.* (1977)).

The presence of drosopterins in the orange spots of males explains how it is possible for the size and number of the spots to be unaffected by the level of carotenoids in the diet (see Kodric-Brown 1989; Grether *et al.* 1999).

(b) Geographic patterns

If drosopterins function as carotenoid mimics and compensate for carotenoid scarcity then the drosopterin and carotenoid contents of the orange spots should be negatively correlated across streams. The mean drosopterin content of the orange spots varied significantly between streams (one-way ANOVA on $\log_{10}$ drosopterin mass, $F_{5,84} = 58.02$, $p < 0.0001$; $\log_{10}$ drosopterin concentration (i.e. mass divided by total orange-spot area), $F_{5,84} = 27.30$, $p < 0.0001$) but the correlation with the mean carotenoid content was strongly positive, rather than negative (figure 1). Orange-spot drosopterin content was also positively correlated, across streams, with foregut carotenoid content (figure 2).

In the subset of males matched for total orange area, males from the high-carotenoid-availability Marianne and Paria streams had significantly more drosopterins in their orange spots than males from the corresponding low-carotenoid-availability streams (table 1). The reverse pattern was observed, however, in the Quare streams, where the stream differences in carotenoid availability were smallest (table 1).

Thus, in general, drosopterin production positively matched, rather than compensated for, population differences in carotenoid availability.

(c) Within-stream patterns

The positive correlation between drosopterins and carotenoids across populations could result from, first, a direct developmental reaction norm linking drosopterin production to carotenoid deposition in individual fishes, second, an indirect reaction norm linking drosopterin production to an environmental cue correlated with carotenoid availability at the population level, or third, genetically fixed population differences in drosopterin production tuned to match the average carotenoid availability (a combination of these mechanisms is also possible). Common garden and diet experiments would be required to distinguish between the second and third mechanisms. Our field data are sufficient to test the first mechanism, however, which predicts positive within-stream correlations between the amounts of carotenoids and drosopterins in the orange spots of individual fishes.
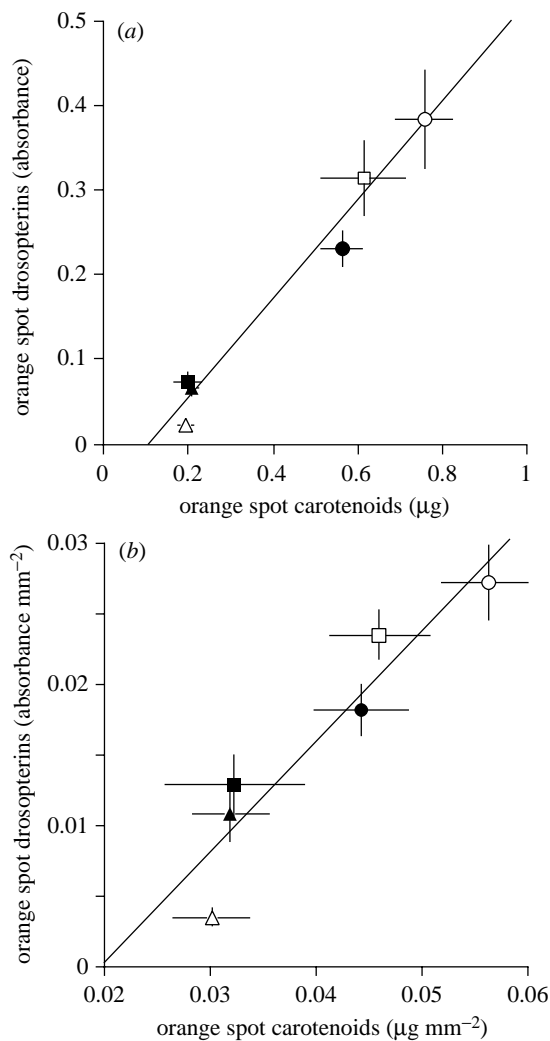
Figure 1. Geographical covariation between drosopterin and carotenoid contents of the orange spots of male guppies in terms of (a) pigment mass ($r = 0.99$, $n = 6$, $p < 0.0001$) and (b) pigment concentration ($r = 0.94$, $n = 6$, $p = 0.0026$). Squares, circles and triangles represent the Marianne, Paria and Quare river basins, respectively, with filled symbols representing the low-carotenoid-availability streams and open symbols representing the high-carotenoid-availability streams. Points represent stream means $\pm$ s.e.m. and the lines are from least-squares regressions. Drosopterin values are shown in absorbance units, instead of micrograms, because the drosopterin extinction coefficient is unknown (absorbance is linearly related to pigment content).

These correlations were significantly positive in the two streams with the highest carotenoid availability but non-significant in the other four streams (table 2). One likely reason for this pattern of correlations is that the upper limit for pigment deposition, set by the total orange-spot area, was reached in the two streams with the highest carotenoid availability and not in the other streams. Consistent with this explanation, pigment content correlated significantly with orange-spot area only in the high-carotenoid-availability streams (table 2). Moreover, all partial correlations between the drosopterin and carotenoid contents of the orange spots, holding orange area constant, were non-significant (table 2). Thus, we have no evidence for a reaction norm linking drosopterin production to carotenoid deposition in individual fishes.
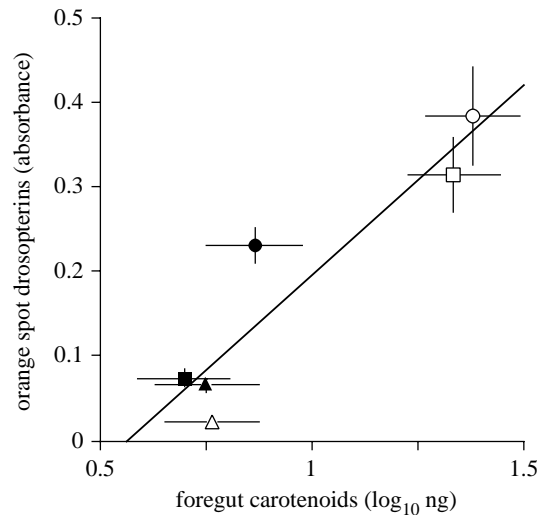
Figure 2. Geographical covariation between the drosopterin content (mass) of the orange spots and carotenoid ingestion, as inferred from foregut contents ($r = 0.93$, $n = 6$, $p = 0.0048$). Symbols are as for figure 1. Orange-spot drosopterin concentration (not shown in this figure) was also significantly correlated with foregut carotenoid content ($r = 0.87$, $n = 6$, $p = 0.02$) (Grether et al. 1999, fig. 2 shows the regression of orange-spot carotenoids on foregut carotenoids, but note that the symbols for Marianne and Paria were erroneously reversed.)

4. DISCUSSION

The discovery of drosopterin pigments in the orange spots of male guppies is not novel from a comparative biochemistry perspective; pteridines have long been known to contribute to the sexual coloration of poeciliid fishes (Goodrich et al. 1941; Henze et al. 1977) and other animals (Needham 1974; Fox 1976). What is surprising is that these pigments have received little, if any, mention in a sexual-selection and signal-evolution context. Handicap theory predicts that the end point of signal evolution is an honest-signalling equilibrium, where the benefits of producing deceptive signals are outweighed by the costs (Zahavi 1977; Grafen 1990; Zahavi & Zahavi 1997). Honesty-enforcing mechanisms proposed specifically for carotenoids include the following: first, that carotenoids are scarce in the environment and, thus, costly for weak foragers to obtain (Endler 1980; Hill & Montgomerie 1994); second, that parasites interfere with carotenoid absorption, metabolism or deposition (Houde & Torio 1992; Thompson et al. 1997; Zahn & Rothstein 1999); third, that carotenoids confer health benefits elsewhere in the body and, thus, are costly to display (Lozano 1994); and fourth, that carotenoids are toxic to ingest (Olson & Owens 1998; but see Møller et al. 2000). But if using carotenoids is costly, what prevents 'cheaters' from using pteridines instead? Do carotenoids have important spectral properties that cannot be mimicked by pteridines? Are the costs of pteridine synthesis prohibitive? Or are species with carotenoid coloration at a stage in signal evolution where the relevant biosynthetic pathways have not yet evolved? This problem can also be framed in simple optimality terms, independent of signaller–receiver coevolution. Assuming that there is an optimal level of

Table 1. *Within river-basin comparison of streams with high and low carotenoid availabilities*

(The values shown are mean differences (s.e.m.) between high-carotenoid-availability (HCA) and low-carotenoid-availability (LCA) streams within river basins. Positive values indicate that the HCA stream mean exceeded the LCA stream mean. Carotenoid and spot-area results, reproduced from Grether *et al.* (1999), are included here for comparison with the drosopterin results. Note that orange-spot-area differences between streams were removed by pairing males with similar orange areas. See § 2b for further details.)

	river basin		
dependent variable	Marianne	Paria	Quare
orange-spot drosopterins ($\log_{10}\mu g$)	0.41 (0.08)[***]	0.19 (0.07)[*]	-0.57 (0.14)[***]
orange-spot carotenoids ($\log_{10}\mu g$)	0.28 (0.08)[**]	0.19 (0.05)[***]	-0.03 (0.08)
foregut carotenoids ($\log_{10}\mu g$)	0.64 (0.15)[****]	0.52 (0.16)[***]	0.01 (0.16)
orange-spot area (mm^2)	0.15 (0.16)	0.20 (0.17)	-0.01 (0.04)

[*]$p < 0.05$, [**]$p < 0.01$, [***]$p < 0.005$, [****]$p < 0.0001$.

Table 2. *Within-stream correlations and partial correlations*

(The values shown are correlations between (a) the mass of drosopterins (D) and carotenoids (C) in the orange spots of male guppies; (b) D and total orange-spot area (A) and (c) C and A; (d) partial correlations between D and C holding A constant; and correlations between (e) the concentrations of drosopterins ([D]) and carotenoids ([C]) in the orange spots of male guppies, (f) [D] and A, and (g) [C] and A. Pigment masses were $\log_{10}$ transformed. All $n = 15$. Results (c) and (g) are reproduced from Grether *et al.* 1999.)

river basin	carotenoid availability	(a) DC	(b) DA	(c) CA	(d) DC.A	(e) [D][C]	(f) [D]A	(g) [C]A
Marianne	high	0.61[**]	0.80[****]	0.77[***]	-0.03	-0.17	0.12	0.08
Marianne	low	0.29	-0.14	0.41	0.38	0.70[***]	-0.69[***]	-0.51[§]
Paria	high	0.68[***]	0.78[****]	0.65[**]	0.36	0.31	0.21	-0.22
Paria	low	-0.1	-0.12	-0.06	-0.10	0.17	-0.49[§]	-0.50[§]
Quare	high	0.42	-0.03	0.55[*]	0.51[§]	0.23	-0.41	-0.29
Quare	low	0.31	-0.18	0.05	0.33	0.55[*]	-0.65[**]	-0.76[****]

[§]$p < 0.1$, [*]$p < 0.05$, [**]$p < 0.02$, [***]$p < 0.005$, [****]$p < 0.001$.

conspicousness, where the benefits (e.g. mate attraction) and costs (e.g. detection by predators) of sexual coloration balance, then relying on condition-dependent carotenoid pigments would appear to be maladaptive if synthesizable alternatives to carotenoids exist.

Since the orange spots of guppies contain both types of pigments, and other relevant aspects of this system are well studied, we were able to formulate a clear prediction of the carotenoid-mimicry hypothesis. Female guppies, in most populations, prefer males with higher orange chroma (Kodric-Brown 1989, 1993; Houde & Torio 1992; Grether 2000) and greater orange area (Houde 1988; Houde & Endler 1990; Endler & Houde 1995; Jirotkul 1999). In streams with low carotenoid availability, there is a trade-off between orange area and the concentration of carotenoids in the spots, i.e. males with larger total orange area have lower carotenoid concentrations (Grether *et al.* 1999). Since carotenoids and drosopterins both contribute positively to the chroma of the orange spots, it follows that males should be selected to produce more drosopterins as carotenoid availability decreases.

We found, however, that males in low-carotenoid-availability streams tended to have less, rather than more, drosopterins in their orange spots than males in high-carotenoid-availability streams (table 1). Across streams, orange-spot drosopterin content was positively correlated

with orange-spot carotenoid content (figure 1) and carotenoid ingestion (figure 2). A complicating factor, which may explain these unexpected results, is that food availability and carotenoid availability are closely linked: unicellular algae appear to be the primary food source and also the primary carotenoid source for guppies (Grether *et al.* 1999, 2001). Guppies grow faster in streams with higher algae availabilities, which indicates that food is limiting (Grether *et al.* 2001). Perhaps the metabolic cost of drosopterin production is sufficiently high that the optimum level of production actually decreases as algae availability decreases (we are not aware of any relevant data on the costs of drosopterin production). If drosopterin production is constrained by food intake in individual males then drosopterins and carotenoids could be viewed as parallel indicator systems, i.e. both types of pigments would improve the value of the orange spots as indicators of male foraging ability and health. Alternatively, fixed population differences in drosopterin production might have evolved in response to differences in average food availability between streams. The latter mechanism is more compatible with our results, given the lack of correlation between drosopterin and carotenoid contents of the orange spots among males in the low-carotenoid-availability streams (table 2). Under both of these scenarios, drosopterins would seem to be an expensive substitute for
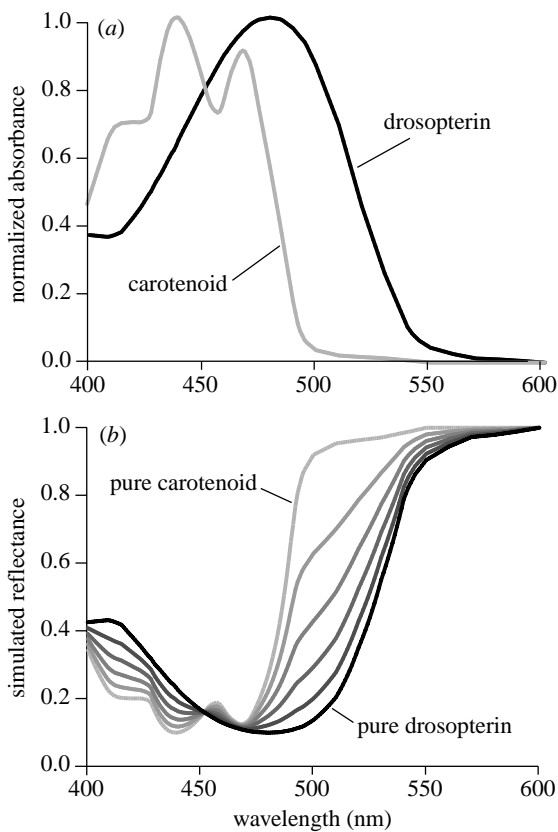
Figure 3. (*a*) Normalized absorbance spectra for carotenoids and drosopterins extracted from orange spots of male guppies. (*b*) Simulated reflectance spectra for different carotenoid to drosopterin ratios. The simulation indicates that the orange spots would appear yellower (to human eyes) as the proportion of carotenoids in the spots increases, and redder as the proportion of drosopterins increases. Absorbance spectra were taken from spectrophotometer scans of individual pigment extracts and normalized to a minimum of 0 and a maximum of 1. Simulated absorbance spectra for different pigment ratios (not shown) were calculated for each wavelength using the formula $A = pC + (1-p)D$, where p is a constant ranging from 0 to 1 and C and D are the normalized carotenoid and drosopterin absorbances. Simulated reflectance spectra were obtained by equating reflectance and transmittance using the formula $T = 10^{-A}$. The following values of p were used in the figure: 0, 0.2, 0.4, 0.6, 0.8 and 1.0. The simulation makes some simplifying and unrealistic assumptions. The absorbance spectra of the pigments in intact skin cells may differ from those measured from pigment extracts. Also, *in vivo*, reflectance and transmittance would be affected by properties of the skin that were not taken into account here.

carotenoids and little threat to the indicator value or honesty of carotenoid coloration.

Another possible explanation for the positive correlation between the carotenoid and drosopterin contents of the orange spots across streams is that female guppies prefer a particular 'hue'. As illustrated in figure 3, changes in the ratio of the pigments should change the shape of the reflectance spectrum and the perceived hue of the orange spots. Under this hypothesis, males would be selected to match actual or expected carotenoid deposition with a complementary quantity of drosopterins, resulting in a positive correlation between the two types of pigments across
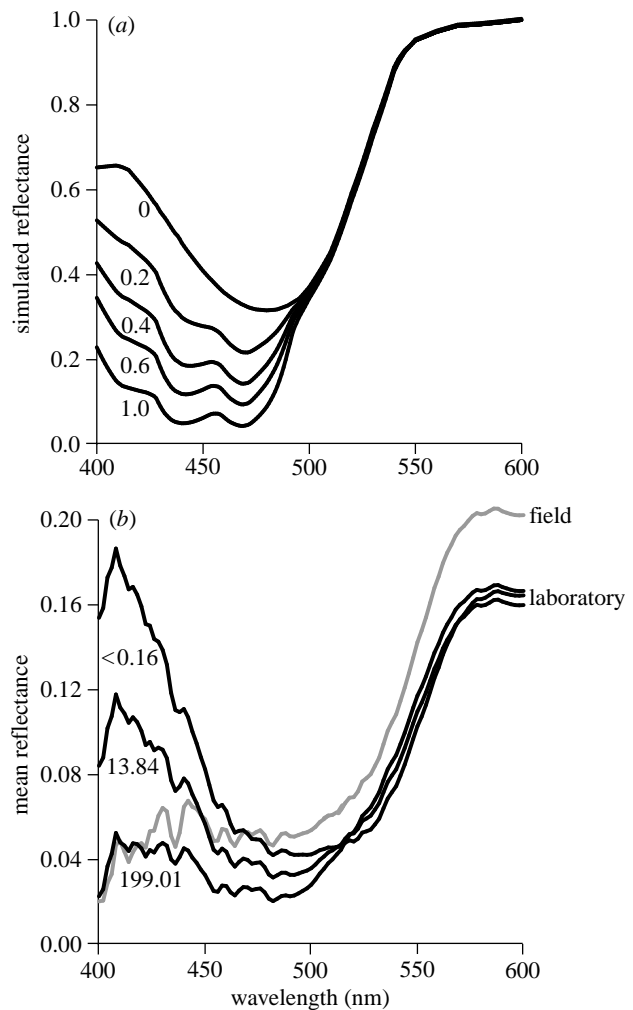
Figure 4. (*a*) Simulation showing the effect on orange-spot reflectance of varying carotenoid content while holding drosopterin content constant. Simulated reflectance spectra were generated as described in the legend for figure 3, except that absorbance spectra were calculated using the formula $A = pC + 0.5D$. The following values of p were used in the figure: 0, 0.2, 0.4, 0.6 and 1.0. (*b*) Mean orange-spot reflectance spectra for guppies in the field (grey line) and laboratory (black lines). The three black lines correspond to different levels of dietary carotenoids (< 0.16 ppm, 13.84 ppm and 199.01 ppm of β-carotene). Second-generation laboratory-born males were raised on these diets from five to eight weeks of age until they were fully mature, and the reflectance spectrum of their orange spots was measured with a spectroradiometer (see Grether (2000) for further details). Only data for the low-carotenoid-availability Paria stream are shown; results for the other five streams were similar with respect to the patterns emphasized here. The number of males (and orange body spots) contributing to the mean reflectance spectra were as follows: field 38 (158); < 0.16 ppm diet 21 (84); 13.84 ppm diet 19 (76) and 199.01 ppm diet 18 (74).

stream means. The seemingly open-ended preference of female guppies for males with higher orange chromas (Kodric-Brown 1989; Grether 2000) could be reconciled with a 'sliding-ratio' preference that favoured increases in pigment content so long as the carotenoid to drosopterin ratio remained above a particular value. This could be tested by varying the carotenoid and drosopterin components of the spots independently and examining the effects

on male attractiveness. A sliding-ratio preference could be a female counter-adaptation to males 'cheating' with drosopterins in a carotenoid-indicator system, or it could reflect a pre-existing sensory bias (see the review by Endler & Basolo 1998).

It is reasonable to consider whether our results could be explained by subtle variation in the spectral environment (water transmission, background or ambient light spectra). Changes in the spectral environment could potentially shift the optimal ratio of drosopterins to carotenoids for maximizing visual contrast and cause drosopterin production to evolve through the sensory-drive process (Endler 1992; see also Reimchen 1989). However, we found that the carotenoid to drosopterin ratio of the orange spots was nearly constant across streams (figure 1). Although the total pigment content of the orange spots varied between streams, this cannot be explained by variation in the spectral environment because carotenoid deposition is constrained by carotenoid availability, as opposed to being free to evolve (Grether *et al.* 1999). Furthermore, it seems improbable that variation in the spectral environment could result in the nearly perfect correlation ($r = 0.99$) between the drosopterin and carotenoid contents of the orange spots across stream means (figure 1).

Our within-stream analyses suggest that the geographical variation in drosopterin production is genetic or linked to an environmental cue correlated with carotenoid availability at the population level, and not the result of a reaction norm linking drosopterin production to carotenoid deposition in individual fishes. If this is so then feeding guppies different amounts of carotenoids should not affect the drosopterin content of their orange spots. Although we do not yet have the data required to test this prediction directly, the analysis presented in figure 4 indicates that the prediction is probably correct. Figure 4*a* shows that when the carotenoid content of the orange spots increases but the drosopterin content is held constant, this should reduce reflectance below 500 nm but have little effect above 500 nm. In contrast, changing the quantities of both types of pigments should alter reflectance across the spectrum (figure 3*b*). Figure 4*b* shows that when male guppies were raised in a common environment on different dietary levels of carotenoids, reflectance of the orange spots below 500 nm decreased as carotenoid levels increased, but reflectance above 500 nm remained fairly constant. Although the simulated and observed reflectance spectra differ in several respects (no doubt in part because of reflectance and transmission properties of the skin not included in the simulation), we view the similarities as further evidence that drosopterin production is not linked directly to carotenoid deposition.

If males benefit from displaying a particular carotenoid to drosopterin ratio but are unable to match drosopterin production directly to carotenoid deposition, what quantity of drosopterins should a male with a given orange-spot area produce? Because orange area is developmentally fixed in guppies (i.e. highly heritable and not affected by carotenoid intake; Kodric-Brown 1989; Houde 1992; Grether 2000) we can make some testable predictions. In carotenoid-limited streams, males with large orange areas cannot deposit more carotenoids in their orange spots than males with small orange areas, and, thus, carotenoid concentration decreases as orange

area increases (see table 2; Grether *et al.* 1999). In these streams, the best strategy would be to produce a fixed total quantity of drosopterins, irrespective of orange area. Consistent with this prediction, in the low-carotenoid-availability streams the amount (mass) of drosopterins in the orange spots was not correlated with either carotenoid mass or orange area (table 2), and the concentration of drosopterins in the orange spots decreased as orange area increased (table 2). Conversely, in carotenoid-unlimited streams, males with large orange areas can deposit more carotenoids in their spots than males with small orange areas (see table 2; Grether *et al.* 1999). In these streams, males with large orange areas should produce more drosopterins than males with small orange areas. Consistent with this prediction, in the two streams with the highest carotenoid availability the quantity of drosopterins in the orange spots was positively correlated with both carotenoid mass and orange area (table 2), and the concentration of drosopterins in the orange spots was not correlated with orange area (table 2). Apparently, guppies use different mechanisms for matching drosopterin production to expected carotenoid deposition in carotenoid-limited and carotenoid-unlimited streams.

The comparative biochemistry of animal coloration may shed light on the selective mechanisms through which coloration evolves. Two recent studies adopting this perspective examined the contribution of carotenoids and melanins to sexual dichromatism (an indirect measure of the intensity of sexual selection) in birds (Gray 1996; Badyaev & Hill 2000), as a means of evaluating the relative importances of indicator versus non-indicator processes in the evolution of avian plumage. Because melanins are endogenously produced as a by-product of amino-acid catabolism (Fox 1976), melanin-based coloration may be less environmentally sensitive, and therefore less likely to reflect the condition of the bearer, than carotenoid-based coloration (for evidence, see McGraw & Hill 2000). If so, melanin-based coloration should be less likely than carotenoid coloration to evolve through the indicator processes of sexual selection. Both studies found that carotenoids contribute more to avian sexual dichromatism than do melanins, after controlling for phylogeny. One interpretation of this result is that sexual dichromatism in birds evolves primarily through the indicator processes of sexual selection. However, carotenoids and melanins differ not only in their origin (exogenous versus endogenous) but also in their spectral properties and conspicuousness. Carotenoids have clear-cut absorption spectra that result in bright saturated colours while melanins have sloping spectra that produce poorly saturated colours with a substantial achromatic (grey) component. Thus, it may be the conspicuousness of carotenoids, and not their exogenous origin, that is responsible for the association between carotenoids and sexual dichromatism. Moreover, melanins, in their various shades of brown, may be better suited than carotenoids for crypsis. Coloured pteridines, on the other hand, are endogenous pigments with spectral properties similar to carotenoids. Both carotenoids and pteridines are present in the skin of guppies and some other poeciliid fishes (*Xiphophorus* spp.; Goodrich *et al.* 1941; Henze *et al.* 1977), suggesting that a valid comparative test for a link between pigment origins and sexual dichromatism may be possible in this group.

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